when administered to rabbits, although they yield hippuric and mandelic acids.

6. Phenacyl alcohol is not reduced to phenylglycol in the rabbit. A small proportion (< 5 %) of it appears to be excreted as phenacyl alcohol glucuronide, which was isolated.

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REFERENCES

Azouz, W. M., Parke, D. V. & Williams, R. T. (1952). *Biochem. J.* **50**, 702.
Carpenter, C. C., Shaffer, C. B., Weil, C. S. & Smyth, H. F. (1944). *J. industr. Hyg.* **26**, 69.
Danishefsky, I. & Willhite, M. (1954). *J. biol. Chem.* **211**, 549.
Eimhjellen, K. E. (1956). *Biochem. J.* **64**, 4P.
El Masri, A. M., Smith, J. N. & Williams, R. T. (1956). *Biochem. J.* **64**, 50.
Kamil, I. A., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 235.
Magat, M. & Maier, N. (1943). *Annual Tables of Constants and Numerical Data*, vol. 13, section 33. Paris: Hermann et Cie.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1958). *Biochem. J.* **68**, 67.
Parke, D. V. & Williams, R. T. (1950). *Biochem. J.* **46**, 236.
Paul, J. (1951). Ph.D. Thesis. University of Glasgow.
Robinson, D., Smith, J. N. & Williams, R. T. (1955). *Biochem. J.* **59**, 153.
Robinson, D. & Williams, R. T. (1955). *Biochem. J.* **59**, 159.
Smith, J. N., Smithies, R. H. & Williams, R. T. (1954*a*). *Biochem. J.* **56**, 317.
Smith, J. N., Smithies, R. H. & Williams, R. T. (1954*b*). *Biochem. J.* **56**, 320.
Smith, J. N., Smithies, R. H. & Williams, R. T. (1954*c*). *Biochem. J.* **57**, 74.
Spencer, H. C., Irish, V. K., Adams, E. M. & Rowe, D. D. (1942). *J. industr. Hyg.* **24**, 295.
Sperber, I. (1948). *J. biol. Chem.* **172**, 441.
Stekol, J. A. (1936). *J. biol. Chem.* **113**, 279.
Wild, F. (1947). *Characterisation of Organic Compounds.* Cambridge University Press.

The Preparation and Properties of Ergothioneine Disulphide

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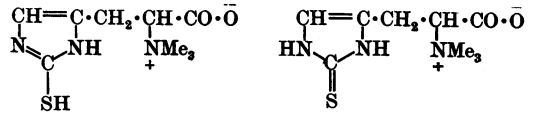
Ergothioneine occurs both intracellularly in blood (Hunter & Eagles, 1927) and extracellularly in seminal plasma (Mann & Leone, 1953), but it has never been shown to occur in either a combined form or in a higher state of oxidation.

Although ergothioneine is usually written in the thiol form (I) it is not a normal thiol compound as it does not give a nitroprusside reaction; neither, however, does it give the blue colour with the Grote reagent (Grote, 1931) typical of thiourea and substituted thioureas (Kjaer & Rubinstein, 1953) (II).



(I) (II)

The chemical reactions of the sulphur in the mercaptoglyoxalines are different from those of normal thiols, in that the sulphur is completely unaffected by boiling 50 % aqueous potassium hydroxide (Barger & Ewins, 1911), but is rapidly cleaved from the glyoxaline ring in the form of sulphate by even weak oxidants such as ferric sulphate (Ashley & Harington, 1930), to yield the corresponding glyoxaline. As a generalization it can be stated that the majority of the 2-mercaptoglyoxalines do not form stable disulphides, sulphenic or sulphinic acids, but this depends on the substituents in the 4 and 5 positions of the glyoxaline ring and there are a few exceptions to this rule. For a complete review of the chemistry of these compounds see Heath (1951). There is only one report of a higher oxidation product of ergothioneine (Barger & Ewins, 1911). By the action of iodine on ergothioneine they obtained black, steel-grey or blue, mixed crystals of a periodide which they stated was that of ergothioneine disulphide. No evidence or analysis in support of this statement was given; it is probable that the product, besides being a periodide, is also iodinated in the 4(5) position, since this halogen substitution readily occurs as was shown in the synthesis of 4-bromo-5-methyl-2-glyoxalyl disuphide (Heath, Lawson & Rimington, 1951).

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Circumstantial evidence, however, that ergothioneine might be catalytically oxidized is to be found in several early papers on the oxidation of cysteine and glutathione. Woodward & Fry (1932) showed that whereas a pure solution of ergothioneine is not oxidized by iodine, under their conditions for the estimation of glutathione, it is oxidized in the presence of glutathione. Pirie (1933) also showed from polarimetric observations that ergothioneine catalysed the hydrogen peroxide oxidation of cysteine.

The biochemical function of ergothioneine is not known, and, although from the organic chemical approach it seemed unlikely that ergothioneine disulphide could exist as a stable compound, we decided to investigate whether ergothioneine disulphide could exist even temporarily as a metabolic intermediary. Some of these results have been briefly reported elsewhere (Heath & Toennies, 1955).

EXPERIMENTAL

By analogy with the chemistry of the mercaptoglyoxalines as a whole, it was obvious that the mildest possible conditions of oxidation would be necessary for the formation of ergothioneine disulphide, and in the first instance the effect of oxygen at various pH levels and in the presence and absence of catalysts was studied. The reactions were followed spectrophotometrically by using a Beckman Model DU spectrophotometer.

The ultraviolet-absorption spectra of ergothioneine and thiourea, 50 μM in water, pH 7, are shown in Fig. 1. The change in wavelength of the maximum in the spectrum of ergothioneine with change of pH is shown in Fig. 2. The spectrum of ergothioneine at all pH values below 9 has a sharp maximum at 258 mμ, log E 4·16; as the pH is increased above 9 the maximum is displaced to shorter wavelengths and becomes progressively broader. The ultraviolet-absorption spectrum of thiourea (Mason, 1954) has a sharp maximum at 236 mμ, log E 4·08, and there is no change in the wavelength of the maximum between pH 2·4 and 12·1. There is no absorption in the spectrum of either compound above 300 mμ at any pH. The pH values of the solutions were determined with a Beckman pH meter. For the ultraviolet measurements corresponding solutions of the buffers were used as blanks.

Action of oxygen on ergothioneine in the presence of glutathione

The presence of an equimolar concentration of reduced glutathione does not affect the ultraviolet spectrum contributed by ergothioneine (50 μM) at wavelengths greater than 230 mμ.

A mixture of 0·01 M-ergothioneine hydrochloride (1 ml.) and 0·01 M-reduced glutathione (2 ml.) was slowly aerated in water for 1·5 hr. in a weighed apparatus; at the end of the aeration period, the solution was made up to the original weight with water and a portion was diluted for ultraviolet measurement. No change in the ultraviolet spectrum of ergothioneine was observed, and the solution still gave a strongly positive nitroprusside test for reduced

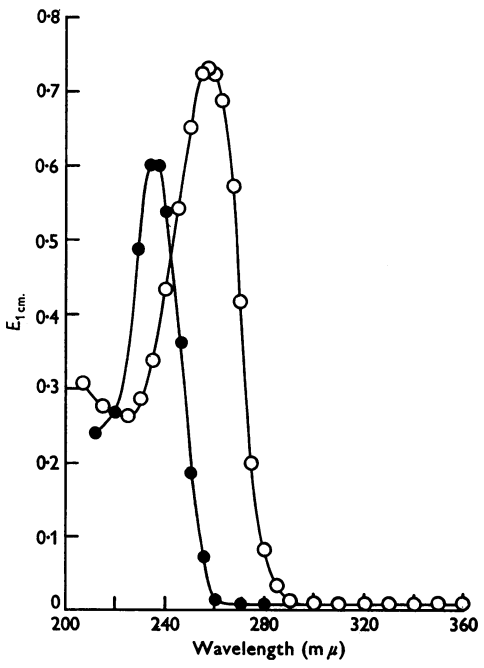

Fig. 1. Ultraviolet-absorption spectrum of (*a*) ergothioneine (○) and (*b*) thiourea (●) (50 μM in water); pH 7.

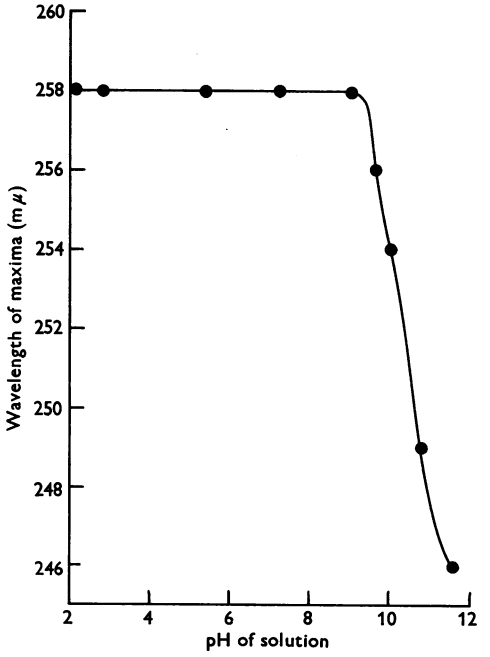

Fig. 2. Relationship between the wavelength (mμ) of the maximum in the ultraviolet-absorption spectrum of ergothioneine and pH.

glutathione. Hence little, if any, oxidation of either ergothioneine or glutathione had occurred.

As glutathione is not oxidized by atmospheric oxygen in the absence of heavy metals at low pH values, but is rapidly oxidized in the presence of copper, this rate increasing with increase in pH (Lyman & Barron, 1937), 5 ml. of 0·01 M-glutathione, 0·5 ml. of 0·001 M-$CuSO_4$ and 0·5 ml. of 10% sodium acetate–1% Na_2CO_3 soln. (pH 10·5) were mixed and aerated under the same conditions. After 1 hr. the solution failed to give the nitroprusside reaction, showing that complete oxidation of the glutathione had occurred. A solution (5 ml.) of 0·01 M-ergothioneine was then aerated in the same way, but after 2 hr. there was no change in the ultraviolet spectrum and oxidation of the ergothioneine had not occurred. On aeration of a mixture of 0·01 M-ergothioneine and glutathione under the same conditions, the glutathione was oxidized but the ergothioneine was not. After standing at room temperature for 60 hr. there was still no change in the ergothioneine spectrum. The solution was adjusted to pH 2 with HCl, and it was then observed that after standing for 24 hr. the ergothioneine ultraviolet maximum at 258 mμ was greatly diminished, although there was no appearance of reduced glutathione (GSH). It seemed therefore that at pH 2, in the presence of copper and oxidized glutathione (GSSG), ergothioneine (ESH) was oxidized but the reaction GSSG + ESH → GSSE + GSH did not take place as there was no corresponding formation of GSH. It was further demonstrated that a similar fall in the ultraviolet maximum was caused when ergothioneine was aerated at pH 2 in the presence of Cu^{2+} ions but in the absence of glutathione.

Effect of Cu^{2+} *ions, hydrochloric acid concentration and cysteine on the oxidation of ergothioneine*

A series of solutions of mM-ergothioneine, in water, 0·01 N-HCl and 0·1 N-HCl, both with and without 10^{-5} M-$CuSO_4$, were made in glass-stoppered tubes. For ultraviolet measurements 0·5 ml. of these solutions was diluted to 10 ml. with water and read against a corresponding blank solution. After the initial reading the solutions were saturated with oxygen and allowed to stand at room temperature. The ultraviolet spectra of fresh dilutions of the reaction mixtures were read daily for 8 days. Then a fresh dilution of 0·5 ml. of the oxidized ergothioneine solution was made with 9·5 ml. of mM-cysteine hydrochloride and the corresponding dilution of cysteine used as the blank in the ultraviolet-spectra measurements. The results of these ultraviolet measurements showed that ergothioneine is not oxidized at all by oxygen in pure aqueous solutions, but that a slow oxidation occurs in acid solution and in the absence of Cu^{2+} ions, as indicated by the change in the spectrum, which could be restored to the original ergothioneine spectrum by the addition of cysteine. The addition of copper to these solutions caused, on long standing, a change in spectrum which was not reversed by the addition of cysteine.

When the same experiment was carried out in 5 N-HCl, no detectable oxidation occurred after 2 hr. in the absence of Cu^{2+} ions, but in the presence of 10 μM-$CuSO_4$ a 20% reduction in the maxima at 258 mμ occurred and the change in spectrum after 24 hr. is shown in Fig. 3. Curve (*a*) is the ultraviolet spectrum of the oxidized ergothioneine. Curve (*b*) is this same oxidized product after treatment with cysteine; this spectrum is identical with the spectrum of ergothioneine. Thus ergothioneine is reversibly oxidizable. The oxidation of ergothioneine by aeration in 5 N-HCl in the presence of Cu^{2+} ions is slow and was not suitable as a preparative method; in one experiment it took 7 days' aeration to complete the oxidation of 100 mg. of ergothioneine when dissolved in 10 ml. of 5 N-HCl, mM in Cu^{2+} ions.

Hydrogen peroxide oxidation of ergothioneine

A series of solutions (4 ml.) of 5 mM-ergothioneine hydrochloride in water, 0·05 N, 0·5 N- and 5 N-HCl, both with and without 10 μM-$CuSO_4$, were made and to each, with rapid mixing, was added 0·4 ml. of 0·05 N-H_2O_2. After standing 30 min., 0·11 ml. of each solution was diluted to 10 ml. (*a*) with water, (*b*) with mM-cysteine hydrochloride, for ultraviolet measurement, and in each the corresponding blank solutions were used. The results given in Table 1 show only the absorption at 258 mμ. As oxidations occurred there was also a rise in the absorption at wavelengths greater than 290 mμ similar to that shown in Fig. 3 for the oxidation of ergothioneine with oxygen. The results after 30 min. show that the largest fall in absorptions at 258 mμ occurred in 5 N-HCl. The original spectrum of ergothioneine was restored by the addition of cysteine, in all cases excepting the reaction in water for 24 hr., which indicated that in the latter case some other non-reversible oxidation had occurred. The reactions were the same in the absence as in the presence of Cu^{2+} ions.

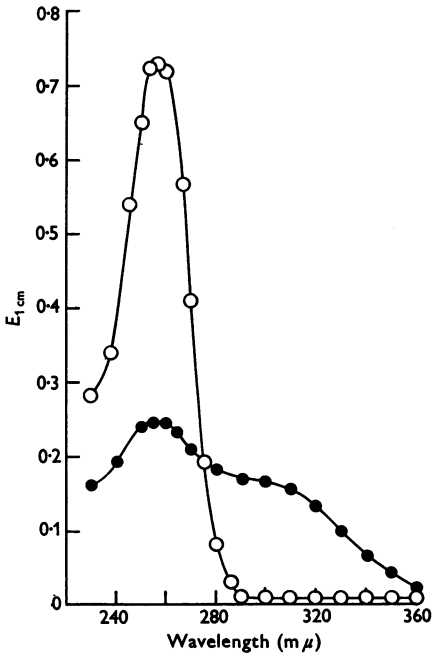

Fig. 3. Ultraviolet-absorption spectra of (*a*) ●, ergothioneine after oxidation with oxygen in 5 N-HCl containing 10 μM-$CuSO_4$, (*b*) ○, the same oxidized product after treatment with cysteine hydrochloride.

Table 1. *Hydrogen peroxide oxidation of ergothioneine in water, 0·05N-, 0·5N- and 5N-HCl in the presence and absence of Cu^{2+} ions and cysteine*

$E_{1\,cm.}$ of 50 μM-ergothioneine at 258 mμ after treatment with H_2O_2.

	After 30 min.		After 24 hr.	
	Cysteine absent	Cysteine present	Cysteine absent	Cysteine present
Water	0·540	0·740	0·397	0·653
Water + Cu^{2+} ions	0·510	0·730	0·404	0·665
0·05N-HCl	0·323	0·735	0·275	0·740
0·05N-HCl + Cu^{2+} ions	0·327	0·738	0·286	0·740
0·5N-HCl	0·267	0·732	0·265	0·732
0·5N-HCl + Cu^{2+} ions	0·256	0·740	0·250	0·735
5N-HCl	0·232	0·745	0·240	0·735
5N-HCl + Cu^{2+} ions	0·235	0·742	0·255	0·730
Control in water without peroxide	0·740	0·740	0·740	0·740

The formation of the disulphide (proof of the disulphide nature of the oxidation product will be given later) can be seen by the development of a yellow colour in the solution both in the oxygen and peroxide oxidations. This colour is formed immediately by peroxide in the 5N-HCl solution, but only develops later with the weaker acid concentration.

Paper chromatography

A systematic search for a satisfactory solvent system which could resolve ergothioneine, ergothioneine disulphide (ESSE), cysteine, cystine and oxidized and reduced glutathione was made. Such a solvent is methyl Cellosolve (2-methoxyethanol; redistilled and peroxide-free) containing 1% (v/v) of 12N-HCl, on either Whatman no. 3MM or no. 1 paper. This solvent, however, does not separate ergothioneine and mercaptohistidine or their corresponding disulphides and we found that propan-2-ol–formic acid (1:1, v/v) was the best solvent system for this separation. R_F values are shown in Table 2. The chromatograms were allowed to run overnight by the ascending–descending technique of Toennies & Kolb (1951), dried at room temperature, suspended for 5 min. in an NH_3 atmosphere in another chromatography tank in order to decompose the disulphide, and blown with a stream of air to remove the ammonia, which gives a background colour, and then developed by dipping in a 0·2% (w/v) ethanolic solution of 2:6-dichloroquinonechloroimide. With ESH and ESSE, brick-red colours develop as soon as the solvent dries from the paper and maximum development occurs after 5 min.; cysteine and reduced glutathione give yellow colours; cystine and oxidized glutathione do not react. Care should be taken not to expose the paper to collidine, lutidine or phenol vapours, and chromatography tanks which have been used previously for these solvents are not satisfactory because they cause the development of a purple background. The atmosphere in some laboratories causes this background to develop slowly but this can be prevented by further developing the chromatogram by dipping in 0·1% (w/v) ninhydrin in anhydrous acetone. The red quinone colours given by ergothioneine are not affected if the ninhydrin colours are allowed to develop overnight at room temperature, but some fading occasionally occurs on heating in the oven. Cysteine and glutathione give the usual ninhydrin reaction superimposed on the yellow quinone colour, and substances such as thiolhistidine and the mixed disulphide of ergothioneine and cysteine (ESSC, see below) give both the red quinone and the purple ninhydrin reaction by this procedure. We used the method of Toennies & Kolb (1951) to photograph the chromatograms first after quinone development and again after the final development with ninhydrin.

Table 2. *R_F values of various sulphur-containing compounds on Whatman no. 3MM paper by the one-dimensional ascending–descending technique of Toennies & Kolb* (1951)

	R_F in	
Substance	Methyl Cellosolve + 1% (v/v) of 12N-HCl	Propan-2-ol–formic acid (1:1, v/v)
Ergothioneine	0·44	0·47
Ergothioneine disulphide	0·09	0·50
Cysteine	0·66	0·45
Cystine	0·33	0·17
Mercaptohistidine	0·41	0·22
Mercaptohistidine disulphide	0·09	0·17
Glutathione	0·52	0·48
Cysteinylergothioneine	0·16	—
Glutathionylergothioneine	0·24	—

Preparation of ergothioneine disulphide

Ergothioneine hydrochloride (106·2 mg., 0·4 m-mole) was dissolved in 5N-HCl (4 ml., resulting concentration 0·1M); to this solution was added from a microburette 2N-H_2O_2 (0·2 ml., freshly prepared and standardized), with rapid stirring. Reaction was immediate, as shown by the formation of the yellow colour, paper chromatography and ultraviolet spectra. The solution was evaporated to dryness over NaOH in a vacuum desiccator and the yellow, very deliquescent, residue was dissolved in anhydrous ethanol (dried by distillation from Mg and I_2). The solution was centrifuged to remove traces of any insoluble ergothioneine hydrochloride and the hydrochloride of ergothioneine disulphide was precipitated by the addition of excess of sodium-dried ether, collected by centrifuging and washed with ether; while still moist with ether, the product was placed in a vacuum desiccator and the ether removed in an anhydrous atmosphere. The compound was purified by several reprecipitations from anhydrous ethanol with ether. The pale-yellow, very deliquescent, amorphous

powder, m.p. 198–200° by micro-hot-plate method, was chromatographically pure. Analysis was by Weiler and Strauss, Oxford (Found: C, 41·0; H, 5·7; N, 15·4; S, 12·1; Cl, 13·5. $C_{18}H_{30}O_4N_6S_2Cl_2$ requires C, 40·8; H, 5·7; N, 15·8; S, 12·1; Cl, 13·4%).

Besides being deliquescent, ergothioneine disulphide dihydrochloride is unstable in water; aqueous solutions, used for chromatography, show a progressive formation of ergothioneine, with a corresponding diminution in the ergothioneine disulphide spot. This decomposition can also be seen spectrophotometrically, the spectrum slowly reverting to that of ergothioneine. This reaction is comparatively slow, complete decomposition of a mM-solution of ergothioneine disulphide dihydrochloride taking 3–5 days. For paper chromatography ergothioneine disulphide dihydrochloride should be dissolved in anhydrous formic acid; such solutions are stable for several weeks when kept in the refrigerator.

The slow decomposition of ergothioneine disulphide dihydrochloride in water is not a simple reduction to ergothioneine, as is the reduction with cysteine or glutathione. The decomposition was followed spectrophotometrically by determining immediately, and thereafter at daily intervals, the absorption at 258 mμ of a solution of ergothioneine disulphide dihydrochloride in water, both before and after reduction with cysteine. When decomposition was complete, the absorption at 258 mμ directly measured was between 60 and 70% of the original ergothioneine value and no further increase was brought about by the addition of cysteine. This slow decomposition of the dihydrochloride becomes immediate if the solution is made alkaline. It has consequently not been possible, so far, to prepare the free base.

Ergothioneine disulphide dihydrochloride gives the typical magenta colour for ergothioneine in the Hunter reaction, but this is equivalent to only 65–70% of the expected colour. When pure ergothioneine disulphide dihydrochloride is subjected to column chromatography either on alumina in water–ethanol–formic acid (Melville & Lubschez, 1953) or on ion-exchange resins (Heath & Wildy, 1956), only ergothioneine is eluted, decomposition having taken place as shown by paper chromatography and differential-ultraviolet spectra with and without cysteine.

Ergothioneine disulphide dihydrochloride is stable in ethanol, formic acid or 5N-HCl solution and has been kept for over a year, without decomposition, when stored as a dry powder.

Formation of the mixed disulphides, ergothioneine–cysteine and ergothioneine–glutathione

Ergothioneine hydrochloride (53·1 mg., 0·2 m-mole) and cysteine hydrochloride (31·5 mg., 0·2 m-mole) were dissolved in 5N-HCl (4 ml.); to this solution was added from a microburette 2N-H_2O_2 (0·2 ml., freshly prepared and standardized) with rapid stirring. There was an immediate formation of a yellow colour, as with the preparation of ergothioneine disulphide, which then rapidly disappeared to leave a colourless solution. The reaction mixture was evaporated to dryness in a vacuum desiccator, extracted with anhydrous ethanol, centrifuged, precipitated with sodium-dried ether and purified by repeated precipitation from dry ethanol with dry ether.

Paper chromatography of the product, applied in formic acid solution, revealed only one spot. Neither ESH, cysteine nor ESSE was detectable with dichloroquinonechloroimide and treatment with ninhydrin showed the absence also of cystine. Thus the only product of the reaction is the single spot (R_F 0·16) which gives both the quinone and the ninhydrin reactions.

Treatment of the mixed disulphide ergothioneine–cysteine (ESSC) with cysteine results in the immediate formation of ergothioneine and cystine,

$$\text{ESSC} + \text{cysteine} \rightarrow \text{ESH} + \text{cystine}.$$

The mixed disulphide is even more unstable in water than ergothioneine disulphide itself and chromatograms of fresh aqueous solutions show the presence of ergothioneine and cystine. No cysteine is formed in the decomposition. This progressive liberation of ergothioneine with time can be clearly demonstrated chromatographically. An attempt to isolate the free base, by treatment with sodium acetate, at an earlier stage in the preparation before this decomposition was known, resulted only in the isolation of cystine.

The mixed disulphide of ergothioneine and glutathione was prepared in an analogous manner. Its chromatographic behaviour (R_F 0·24) was similar to that of ESSC when applied in formic acid solution.

The hydrochlorides of ergothioneine–cystine and ergothioneine–glutathione are both colourless, deliquescent, amorphous powders, soluble in ethanol. Neither melts below 300°.

Reaction between ergothioneine disulphide and cystine

Solutions (0·05M in 2N-HCl) of ergothioneine disulphide dihydrochloride and cystine were mixed and kept at 20° for 4 days.

No formation by molecular rearrangement of the mixed disulphide ergothioneine–cysteine could be demonstrated (paper chromatography and ultraviolet spectrum).

DISCUSSION

It has been established that ergothioneine can be oxidized under suitable conditions to the disulphide form, and that this oxidation is reversible: $2\text{ESH} \rightleftharpoons \text{ESSE}$. In the physiological pH range, the equilibrium form is, as far as can be detected, completely in the reduced state. It is only in strongly acid solution, in which the disulphide is more stable, that both forms can exist together.

Evidence for this was discovered during the chromatographic part of the experimental work; we made stock solutions, in formic acid, of ergothioneine and ergothioneine disulphide dihydrochloride for use as markers; whereas the disulphide was stable, and did not decompose to ergothioneine, the solution of ergothioneine was slowly oxidized to the disulphide form, the formic acid solution eventually giving both ergothioneine and ergothioneine disulphide spots on the chromatogram. The reverse is true in aqueous solution; ergothioneine is completely stable but the disulphide decomposes to the free thiol form.

The properties of ergothioneine and thiourea disulphides and mixed disulphides with cysteine are similar but not identical (cf. Toennies, 1937; Pirie, 1933). No evidence for the liberation of free sulphur on decomposition of ergothioneine disulphide was found, whereas dithioformamidine readily decomposes in this manner. Likewise the analogue of the reaction between cystine and dithioformamidine to yield the mixed disulphide *S*-guanylthiocysteine could not be demonstrated between ergothioneine disulphide and cystine, even in the presence of free ergothioneine to catalyse the reaction.

However, the formation of mixed disulphides was possible owing to the fact that the oxidation of cysteine in strongly acid solution by hydrogen peroxide is comparatively slow (Toennies & Gallan, 1939), whereas the oxidation of ergothioneine is extremely rapid; this could be seen by the fleeting formation of the yellow colour due to ergothioneine disulphide formation, just after the addition of the peroxide. Thus when a mixture of cysteine and ergothioneine is oxidized in strongly acid solution by peroxide, the first very rapid reaction is $2ESH + H_2O_2 \rightarrow ESSE + 2H_2O$. But the reaction of cysteine and ergothioneine disulphide (ESSE + 2 cysteine $\rightarrow$ 2ESSC) is also extremely rapid and this results in the formation of ergothioneine–cysteine mixed disulphide. Evidence in support of this mechanism is given by the chromatograms of the reaction product, which does not contain any of the original compounds, ergothioneine or cysteine, neither does it contain any of their separate oxidation products, cystine or ergothioneine disulphide, all of which are clearly resolved by the solvent and developing reagents. This reaction mechanism also explains the catalysis of the peroxide oxidation of cysteine in acid solution by ergothioneine.

Owing to the instability of ergothioneine disulphide, it is not possible at present to detect its occurrence in biological systems. In blood ergothioneine always occurs in the presence of a large excess of reduced glutathione, which would maintain it in the reduced form. Even if this were not so, the usual methods of isolation from aqueous extracts by either ion-exchange resins or alumina chromatography would themselves bring about the reduction.

The fact that only 60–70% of the theoretical amount of ergothioneine is detected by ultraviolet absorption or Hunter colour reaction after the decomposition of the disulphide in water indicates that the mode of decomposition is not a simple reduction but probably procedes via a hydrolytic fission to the sulphenic acid,

$$ESSE + H_2O \rightarrow ESH + ESOH,$$

which then decomposes further

$$2ESOH \rightarrow ESH + ESO_2H.$$

The sulphinic acids of the mercaptoglyoxalines are very unstable and readily lose sulphur dioxide (Balaban & King, 1937). It must be emphasized that this mechanism is purely hypothetical and no evidence for the existence of the sulphenic acid has been sought.

The reaction of ergothioneine with 2:6-dichloroquinonechloroimide has been found to be by far the most satisfactory means of chromatographic detection. Colour forms only in neutral or faintly alkaline solution, and ergothioneine disulphide dihydrochloride as such gives only a very weak and slow reaction. After neutralization with ammonia the reaction is immediate owing to the decomposition of the disulphide to ergothioneine.

Mercaptohistidine also forms a disulphide which can be separated chromatographically, with methyl Cellosolve–HCl. This compound, like mercaptohistidine itself, gives both the red quinone reaction, after treatment with ammonia, and a ninhydrin reaction. Its properties are analogous to those of ergothioneine disulphide.

At an early stage in this work attempts were made to prepare ergothioneine disulphide by both iodine and permanganate oxidations. Neither method was successful. It is possible that these or other oxidants could be used, but owing to their high ultraviolet absorption it was not possible to follow the oxidation spectrophotometrically, and owing to the lability of ergothioneine disulphide none was isolated.

The fact that it has now been established that ergothioneine can be catalytically oxidized by molecular oxygen or non-catalytically by hydrogen peroxide might be significant in relation to its biochemical function; it might act as a very transitory proton donor and acceptor.

SUMMARY

1. Ergothioneine can be oxidized slowly to the disulphide form by oxygen in the presence of copper and in strongly acid solution but not in neutral or alkaline solution. The rate of oxidation can be followed spectrophotometrically.

2. Ergothioneine is rapidly oxidized to the disulphide form by the equivalent amount of hydrogen peroxide in strongly acid solution in the absence of copper.

3. This oxidation is reversible. Cysteine or reduced glutathione causes immediate reduction.

4. Ergothioneine disulphide dihydrochloride has been isolated, m.p. 198–200°. It is stable when dry or in strongly acid solution. It is unstable in water or in alkaline solution. Its properties have been described.

5. A paper-chromatographic technique is given for the separation and detection of the reduced and oxidized forms of ergothioneine, cysteine and glutathione.

6. Mixed disulphides of ergothioneine with cysteine and glutathione have been prepared.

The greater part of the work described in this paper was carried out in America but it was completed in England, and one of us (H.H.) wishes to thank Mr R. Boardman for technical assistance in the final stages and Burroughs Wellcome Ltd. for a generous gift of ergothioneine.

REFERENCES

Ashley, J. N. & Harington, C. R. (1930). *J. chem. Soc.* p. 3586.
Balaban, I. M. & King, H. (1937). *J. chem. Soc.* p. 1858.
Barger, G. & Ewins, A. J. (1911). *J. chem. Soc.* **99**, 2336.
Grote, I. W. (1931). *J. biol. Chem.* **93**, 25.
Heath, H. (1951). Ph.D. Thesis, University of London.
Heath, H., Lawson, A. & Rimington, C. (1951). *J. chem. Soc.* p. 2222.
Heath, H. & Toennies, G. (1955). *Proc. 3rd Int. Congr. Biochem., Brussels*, p. 4.
Heath, H. & Wildy, J. (1956). *Biochem. J.* **64**, 612.
Hunter, G. & Eagles, B. A. (1927). *J. biol. Chem.* **72**, 123.
Kjaer, A. & Rubinstein, K. (1953). *Nature, Lond.*, **171**, 840.
Lyman, C. M. & Barron, E. S. G. (1937). *J. biol. Chem.* **121**, 275.
Mann, T. & Leone, E. (1953). *Biochem. J.* **53**, 140.
Mason, S. F. (1954). *J. chem. Soc.* p. 2071.
Melville, D. B. & Lubschez, R. (1953). *J. biol. Chem.* **200**, 275.
Pirie, N. W. (1933). *Biochem. J.* **27**, 1181.
Toennies, G. (1937). *J. biol. Chem.* **120**, 297.
Toennies, G. & Gallan, T. P. (1939). *J. biol. Chem.* **129**, 481.
Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
Woodward, G. E. & Fry, E. G. (1932). *J. biol. Chem.* **97**, 465.

The Purity of Thyroglobulin Isolated from Normal and Carcinomatous Thyroid Tissue on one Patient by Fractional Salting-out with Ammonium Sulphate

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Thyroglobulin was prepared (Stanley, 1956) by the method of Derrien, Michel & Roche (1948) from normal and carcinomatous thyroid tissue of a patient (Mrs Ru.) with a follicular carcinoma of the thyroid gland. The patient had received a tracer dose of radio-iodide 2 days before thyroidectomy. Differences in the distribution of ^{131}I between the fractions obtained during the preparation of pure thyroglobulin have been reported and discussed (Stanley, 1956). The homogeneity and question of identity of the thyroglobulins (Ru_N and Ru_A) from normal and carcinomatous thyroid tissue have now been investigated by electrophoresis and by an immunological test.

MATERIAL AND METHODS

The preparation of Ru_N and Ru_A was described previously (Stanley, 1956). Solutions [1% (w/v); 2·5 ml.] of the two proteins in 0·05M-veronal buffer (pH 8·6, I 0·05) were subjected to electrophoresis in the 2 ml. cell of a Perkin–Elmer apparatus, model 38, after dialysis against the buffer at 0–1° for at least 16 hr. This analysis was repeated with 0·4% solutions of both proteins.

The solutions of Ru_N and Ru_A were recovered from the electrophoresis cells and used for immunological analysis by the method of Ouchterlony (1948). Antibodies against Ru_A were prepared in a rabbit.

RESULTS

Figs. 1 and 2 represent the electrophoretic schlieren patterns obtained with 1% (w/v) solutions of Ru_N and Ru_A respectively, at the times indicated on the figures. Both proteins contain a single electrophoretic component, but differ significantly in mobility. The mobilities in the descending (μ_D) and ascending (μ_A) limbs were as follows: Ru_N: μ_D $5{\cdot}38 \times 10^{-5}$, μ_A $5{\cdot}85 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$; Ru_A: μ_D $4{\cdot}97 \times 10^{-5}$, μ_A $5{\cdot}44 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$. The difference in mobility was confirmed in a second run with 0·4% solutions of the proteins, when the following mobilities were determined: Ru_N: μ_D $5{\cdot}53 \times 10^{-5}$, μ_A $5{\cdot}71 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$; Ru_A: μ_D $4{\cdot}91 \times 10^{-5}$, μ_A $5{\cdot}09 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$.

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